


Glucose Homeostasis in Obese Women Is Not Associated to Unacylated Ghrelin Plasma Levels

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ABSTRACT

INTRODUCTION: Unacylated ghrelin (UAG) is the major form of circulating ghrelin. Initially considered as a nonfunctional peptide, soon after, UAG has been associated to an insulin sensitizing action and to a negative action on energy balance. The aim of this study was to analyze the association between the serum levels of UAG and glucose metabolism parameters in obese women, independently from eventual influence of anthropometrics.

METHODS: One hundred lean and 254 obese Caucasian women were studied. Each woman was characterized for anthropometrics, fasting glucose, insulin, HbA1c, and UAG. In addition, obese women were subjected to a classic oral glucose tolerance test (oGTT) to assess glucose and insulin at 120 minutes. Insulin resistance was assessed by the homeostasis model assessment (HOMA-IR). Obese women were classified in 3 glycemic status subgroups (normoglycemia, prediabetes, and diabetes) according to HbA1c and to fasting and oGTT glucose values.

RESULTS: In comparison with the lean group, significantly lower levels of UAG were observed in obese women. However, no significant difference was observed through obesity classes I to III. UAG levels were not significantly different among glycemic status subgroups and did not show any association with glucose, insulin, HOMA-IR, or HbA1c.

CONCLUSIONS: Although anthropometry can influence the level of the unacylated form of ghrelin, UAG plasma levels do not associate to glucose homeostasis parameters.

KEYWORDS: Unacylated ghrelin, obesity, insulin resistance, dysglycemia

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Introduction

Ghrelin is an orexigenic peptide hormone composed by 28 amino acids initially discovered as an endogenous ligand for growth hormone secretagogue receptor 1a (GHSR-1a).¹ Although predominantly expressed at the stomach, it also can be expressed in low levels at the liver, pancreas, kidney, lung, gut, heart, and central nervous system.^{1–5} This hormone circulates in the bloodstream mainly as unacylated ghrelin (UAG) with the acylated form of ghrelin (AG) corresponding to no more than 10% to 20% of the hormone.⁴ Acylation process is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT) as esterification of the serine-3 residue of the peptide molecule with n-octanoic acid.^{1,6,7} However, it still remains unclear whether UAG represents a precursor or a degradation product of the acylated hormone.^{4,8}

Originally considered as a nonfunctional peptide, UAG has been suggested to have a physiological activity similar to, but distinct from AG.^{9–11}

In addition to its ability to stimulate growth hormone secretion, AG can also stimulate food intake, adipogenesis, and interfere in glucose homeostasis.^{11–13} In what concerns UAG, it seems to induce a negative energy balance,¹⁴ and to have an insulin sensitizing action, suppressing hepatic glucose production¹⁵ and decreasing circulating levels of insulin.¹⁰ The mechanisms underlying those actions are still unclear.

Several authors suggested that UAG could be metabolically active by counteracting the effects of AG on insulin secretion and glucose metabolism in healthy humans.^{10,11,16} Those authors studied the interaction of the combined administration of AG and UAG in normal young volunteers and have found that AG administration induced an increase in glucose plasma levels, while UAG administration alone had no effects on glucose and insulin levels. However, they observed that the combination of the two ghrelin peptide forms diminished the observed effect of AG on glucose values. Despite such findings, others observed that UAG



(administered alone or in combination with AG to healthy humans) did not alter insulin secretion, insulin sensitivity, or glucose metabolism.¹¹

Recent data suggest that UAG has inherent activities in physiological and pathophysiological situations that are independent of AG action.^{17,18} Some authors suggest that UAG should be considered as a separate hormone, that in certain situations act on its own receptor, while in others share with AG a receptor different from the GSHR-1a. These actions could support or antagonize AG functions.¹⁸

Therefore, data are contradictory in human studies, where some of them report a positive relationship between UAG and insulin sensitivity^{2,19,20} and others do not.²¹ Considering this uncertainty, more studies should be conducted to increase the knowledge about AG and UAG effect on carbohydrate metabolism.

The aim of this study was to assess a possible association between UAG levels and parameters of carbohydrate metabolism in obese women, independently from an eventual interference of anthropometrics.

Methodology

Subjects

The study included 254 obese (body mass index [BMI] ≥ 30 kg/m²) Caucasian women, who attended the obesity outpatient clinic at Curry Cabral Hospital—C.H.U.L.C. (Lisbon, Portugal). Their age ranged from 18 to 50 years. The control group consisted on 100 lean (BMI ranging from 18.5 to 24.9 kg/m²) Caucasian women matched for age. All women, obese and lean groups, referred a less than 10% variation of their body weight in the previous year, had a premenopausal status, and were not pregnant or had been pregnant in the precedent 12 months. We considered only women who reported no previous diagnosis of any acute/chronic health condition (including previous diabetes/prediabetes), with the exception of obesity for the obese group. No woman was on any pharmacological regimen (except for oral contraceptives) or took any sporadic drug in the previous 7 days before the blood sample collection.

Obese women were stratified in obesity classes according to BMI (assessed as the ratio between body weight [in kilograms] and the square of height [in meters]): class I obesity ($30.0 < \text{BMI} < 34.9$ kg/m²), class II obesity ($35 < \text{BMI} < 39.9$ kg/m²), and class III obesity (>40 kg/m²).²²

In what concerns glycemic status, women were classified in normoglycemia, prediabetes, and diabetes according to the American Diabetes Association criteria, based on HbA1c and on fasting and 2-hour oGTT glucose levels. Normoglycemia was considered if fasting glucose < 100 mg/dL, 2-hour oGTT glucose < 140 mg/dL, and HbA1c $< 5.7\%$. Prediabetes was considered if fasting glucose ranged from 100 to 125 mg/dL, 2-hour oGTT glucose ranged from 140 to 199 mg/dL, or HbA1c ranged from 5.7% to 6.4%. If fasting glucose ≥ 126 mg/dL,

2-hour oGTT glucose ≥ 200 mg/dL, or HbA1c $\geq 6.5\%$ diabetes was considered.²³

The study was conducted after the approval of the institutional scientific and ethical boards according to the standards of the Declaration of Helsinki. In addition, an informed consent was signed by each participant.

Clinical evaluation

Each woman was characterized for total body weight, height, BMI, waist and hip circumferences, and waist-to-hip ratio. The body fat mass (absolute value and fat mass percentage of total body weight) was assessed by bioelectrical impedance (Tanita TBF-300A, Tanita Europe B.V., Hoofddorp, The Netherlands).

Blood sample collection and measurements

A venous blood sample was collected from patients and controls early in the morning, after an overnight fasting (of at least 10 hours), to avoid potential confounding with hormonal rhythmicity. A classic 75 g oral glucose tolerance test (oGTT) was done to every obese woman in order to assess plasma glucose at the second hour. Serum samples were obtained to assess glucose, glycated hemoglobin (HbA1c), insulin and UAG, using low speed centrifugation, stored at a -80°C ultra-freezer, and thawed just before each assay. Glucose was determined by automated chemistry analyzer (Vitros 5.1 FS Chemistry System, Ortho-Clinical Diagnosis Inc, Rochester, NY, USA). Insulin concentration was assessed by a chemiluminescent immunometric technique (IMMULITE 2000 Immunoassay System, Siemens Healthcare Diagnostics, Camberley, UK). HbA1c was assessed by high performance liquid chromatography (Bio-Rad Laboratories GmbH, Munich, Germany). Serum UAG concentrations were measured by enzyme-linked immunosorbent assay following the manufacturer's instructions (R&D Systems Inc, Minneapolis, MN, USA). Insulin resistance was assessed by the homeostasis model assessment (HOMA-IR) using the following formula: insulin ($\mu\text{U}/\text{mL}$) \times glucose (mg/dL) / 405.²⁴

Statistical analysis

Data were analyzed considering lean ($n=100$) and obese ($n=254$) women subgroups. Obese women were stratified in class I obesity ($n=39$), class II obesity ($n=65$) and class III obesity ($n=150$). Obese women were also stratified into 3 glycemic status subgroups: normoglycemia ($n=165$), prediabetes ($n=63$), or diabetes ($n=19$) in order to search for differences in UAG levels.

Lean, obese, and total groups were characterized as mean (standard deviation) when normal distribution is verified or median (IQR) otherwise. Pearson's correlation coefficients (r) were computed to analyze the relation between 2 variables and Spearman's correlation was used whenever normal distribution

Table 1. Characterization of lean and obese women groups (descriptive statistics are displayed as mean [SD] whenever normal distribution is verified [bold values] and by median [IQR] otherwise).

	TOTAL	LEAN WOMEN	OBESE WOMEN	P VALUE ^a
N	354	100	254	
Age (years)	34.4 (8.4)	34.2 (8.4)	34.6 (8.3)	.702
BMI (kg/m ²)	41 (9)	21.4 (1.7)	41 (8.7)	<.001
Total body weight (kg)	105.1 (22.9)	56.0 (5.3)	105 (22.6)	<.001
Total body fat (kg)	49.1 (17.8)	14.3 (3.6)	49 (17.7)	<.001
Percentage of body fat (%)	46.8 (7.5)	25.3 (4.7)	46.5 (5.2)	<.001
Waist circumference (cm)	115 (18)	71 (7)	115 (18)	<.001
Hip circumference (cm)	131.5 (17)	97 (5)	131.3 (12.8)	<.001
Waist-to-hip ratio	0.87 (0.05)	0.74 (0.05)	0.87 (0.08)	<.001
Unacylated ghrelin (pg/mL)	178.2 (161.6)	350.2 (251.9)	180 (163.4)	<.001
Fasting Insulin (μUI/mL)	13 (11)	5.4 (4.3)	13 (11)	<.001
Fasting glucose (mg/dL)	83 (0.2)	81 (7.1)	83 (16)	<.001
2nd-hour oGTT glucose (mg/dL)	-	-	100 (34)	-
HOMA-IR	2.55 (2.4)	1.1 (0.9)	2.5 (2.4)	<.001
HbA1c (%)	5.3 (0.6)	5.28 (0.28)	5.3 (0.6)	<.001

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment; IQR, interquartile range; oGTT, oral glucose tolerance test.

^aParametric *t* test for 2 independent samples.

was not verified. To control the effects of BMI and waist circumference on the metabolic parameters (fasting insulin, fasting glucose, HOMA-R and HbA1c), partial correlations were computed to evaluate relations between UAG and metabolic parameters. To evaluate the difference between mean values in 2 independent samples, a Student *t* test was used (or the non-parametric alternative Mann-Whitney test whenever the applicable conditions were not verified). Kruskal-Wallis test was used to compare differences among subgroups. To compare distributions of the UAG between obese subgroups or between glycemic subgroups, we have constructed boxplots. A significance level (*P*) of .05 was assumed. All analyses were conducted using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) and the open source R version 3.0.2 software.

Results

Baseline characterization and comparison analysis of lean and obese women groups

Significant differences between lean and obese women groups were verified for all anthropometric, hormonal, and metabolic parameters, namely UAG mean values. UAG was significantly lower in the obese group ($P < .001$) (Table 1).

Despite a decrease in median (IQR) UAG levels in class III obesity compared with class I and II (217 [212.77] pg/mL in

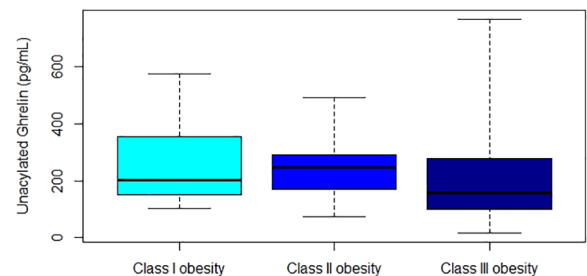


Figure 1. Unacylated ghrelin boxplots in obesity classes: class I obesity ($n=39$), class II obesity ($n=65$), and class III obesity ($n=150$).

class I obesity; 240.47 [156.78] pg/mL in class II obesity; 148.55 [164.82] pg/mL in class III obesity), no significant differences were found among subgroups (Kruskal-Wallis statistics = 11.875, $P = .003$; Figure 1).

To identify possible direct associations between UAG and anthropometric/biochemical parameters, a correlation analysis was performed for the whole population, lean and obese groups (Table 2). No correlation was found between UAG and age either when we considered classes of obesity or glycemic status neither for lean women or when we considered all together. We found a significant inverse correlation between UAG levels and BMI in obese women ($r = -0.187$; $P = .013$). Spearman correlation between UAG and glucose, insulin, HbA1c, and HOMA-IR remained not significant even when we have

Table 2. Spearman correlation coefficients between UAG and anthropometric and hormonal parameters in total, lean and obese women.

VARIABLE	LEAN WOMEN N = 100	OBESE WOMEN N = 254	TOTAL N = 354
BMI (kg/m ²)	-0.058 (<i>P</i> = .580)	-0.187 (<i>P</i> = .013)	-0.236 (<i>P</i> < .001)
Total body weight (kg)	-0.124 (<i>P</i> = .23)	-0.092 (<i>P</i> = .165)	-0.231 (<i>P</i> < .001)
Total body fat (kg)	-0.026 (<i>P</i> = .783)	-0.113 (<i>P</i> = .089)	-0.214 (<i>P</i> < .001)
Percentage of body fat (%)	0.029 (<i>P</i> = .800)	-0.121 (<i>P</i> = .069)	-0.230 (<i>P</i> < .001)
Waist circumference (cm)	-0.093 (<i>P</i> = .372)	-0.083 (<i>P</i> = .214)	-0.218 (<i>P</i> < .001)
Hip circumference (cm)	-0.078 (<i>P</i> = .453)	-0.064 (<i>P</i> = .334)	-0.212 (<i>P</i> < .001)
Waist-to-hip ratio	-0.079 (<i>P</i> = .447)	-0.048 (<i>P</i> = .471)	-0.181 (<i>P</i> = .001)
Fasting insulin (mg/dL) ‡	-0.022 (<i>P</i> = .835)	-0.045 (<i>P</i> = .493)	-0.001 (<i>P</i> = .979)
Fasting insulin (mg/dL) (not adjusted correlations)	-0.049 (<i>P</i> = .637)	-0.042 (<i>P</i> = .520)	-0.137 (<i>P</i> = .012)
Fasting glucose (mg/dL) ‡	0.026 (<i>P</i> = .812)	-0.023 (<i>P</i> = .725)	0.005 (<i>P</i> = .930)
Fasting glucose (mg/dL) (not adjusted correlations)	0.046 (<i>P</i> = .374)	-0.053 (<i>P</i> = .411)	-0.048 (<i>P</i> = .374)
2nd-hour oGTT glucose (mg/dL) ‡	N.A.	-0.020 (<i>P</i> = .762)	N.A.
2nd-hour oGTT glucose (mg/dL) (not adjusted correlations)	N.A.	-0.022 (<i>P</i> = .734)	N.A.
HOMA-IR ‡	-0.038 (<i>P</i> = .726)	-0.034 (<i>P</i> = .601)	0.004 (<i>P</i> = .937)
HOMA-IR (not adjusted correlations)	-0.036 (<i>P</i> = .372)	-0.040 (<i>P</i> = .538)	-0.136 (<i>P</i> = .013)
HbA1c (%) ‡	0.188 (<i>P</i> = .078)	0.021 (<i>P</i> = .743)	0.070 (<i>P</i> = .205)
HbA1c (%) (not adjusted correlations)	0.183 (<i>P</i> = .082)	-0.051 (<i>P</i> = .426)	0.000 (<i>P</i> = .998)

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment; oGTT, oral glucose tolerance test; UAG, unacylated ghrelin. Spearman correlation coefficients between UAG and anthropometric and hormonal parameters and partial correlations (‡) between UAG and metabolic parameters in Obese, Lean and Total (Obese + Lean) women. Partial correlations. Significant *P* values are in bold. Data not available (N.A.).

adjusted to BMI or waist circumference when partial correlations were computed, in each group of women.

It was also computed correlation coefficients between UAG and anthropometric, hormonal, and metabolic parameters in each of the 3 obese stratified subgroups (Table 3) and all values revealed weak correlations, meaning that there is no association between UAG and all the analyzed parameters.

Unacylated ghrelin and glycemic status in obese women

Obese women were classified in 3 glycemic status subgroups (normoglycemia, prediabetes, and diabetes) according to the American Diabetes Association criteria, based on HbA1c and on fasting and 2-hour oGTT glucose levels. One hundred sixty-five obese women were classified as normoglycemic, 63 as prediabetic, and 19 as diabetic. No significant difference was found in UAG levels among normoglycemic, 184.04 (162.51) pg/mL, prediabetic, 154.66 (179.28) pg/mL, and diabetic, 206.79 (160.13) pg/mL, obese women (*P* = .398; Figure 2).

It was also computed correlation coefficients between UAG and anthropometric, hormonal, and metabolic parameters in the 3 glycemic status subgroups (Table 4); there is no correlation between UAG and any specific metabolic/hormonal parameter in the diabetic or in the prediabetic subgroup of patients.

All lean women were classified as normoglycemic based on fasting glucose and on HbA1c (they were not subjected to an oGTT).

Discussion

Originally considered as a nonfunctional peptide, UAG potential effect has been explored. Although available data are still contradictory, an increasing number of studies suggest that UAG can counteract the metabolic effect of AG.^{5,10} If such assumption would be true, UAG could be a good candidate for the future treatment of metabolic disorders such as obesity and diabetes.^{16,25} The main objective of the present study was to assess an eventual association of UAG plasma levels with glucose homeostasis in obese women, independently from an eventual interference of adiposity.

Several studies have shown that obese individuals have lower UAG levels than lean subjects, suggesting that obesity

Table 3. Correlation coefficients between UAG and anthropometric, hormonal, and metabolic parameters in obesity classes.

VARIABLE	CLASS I OBESITY N=39	CLASS II OBESITY N=65	CLASS III OBESITY N=150
BMI (kg/m ²)	0.140 (<i>P</i> = .402)	-0.290 (<i>P</i> = .017)	0.010 (<i>P</i> = .905)
Total body weight (kg)	0.068 (<i>P</i> = .684)	-0.100 (<i>P</i> = .420)*	-0.013 (<i>P</i> = .881)
Total body fat (kg)	0.080 (<i>P</i> = .634)	-0.168 (<i>P</i> = .174)*	-0.053 (<i>P</i> = .531)
Percentage of body fat (%)	0.126 (<i>P</i> = .452)	-0.234 (<i>P</i> = .057)*	-0.024 (<i>P</i> = .773)
Waist circumference (cm)	0.098 (<i>P</i> = .558)	-0.241 (<i>P</i> = .050)	-0.016 (<i>P</i> = .849)
Hip circumference (cm)	0.225 (<i>P</i> = .174)	-0.046 (<i>P</i> = .709)*	0.047 (<i>P</i> = .575)
Waist-to-hip ratio	0.151 (<i>P</i> = .367)	-0.338 (<i>P</i> = .005)	-0.043 (<i>P</i> = .606)
Fasting insulin (mg/dL)‡	0.075 (<i>P</i> = .678)	0.177 (<i>P</i> = .169)*	-0.104 (<i>P</i> = .222)
Fasting insulin (mg/dL) (not adjusted correlations)	0.257 (<i>P</i> = .124)	0.099 (<i>P</i> = .436)*	-0.061 (<i>P</i> = .473)
Fasting glucose (mg/dL)‡	-0.082 (<i>P</i> = .651)	0.025 (<i>P</i> = .849)	-0.014 (<i>P</i> = .870)
Fasting glucose (mg/dL) (not adjusted correlations)	0.056 (<i>P</i> = .740)	-0.142 (<i>P</i> = .264)	-0.014 (<i>P</i> = .871)
HOMA-IR ‡	0.026 (<i>P</i> = .885)	0.141 (<i>P</i> = .275)*	-0.075 (<i>P</i> = .379)
HOMA-IR (not adjusted correlations)	0.256 (<i>P</i> = .127)	0.032 (<i>P</i> = .802)*	-0.046 (<i>P</i> = .590)
HbA1c (%) ‡	-0.036 (<i>P</i> = .844)	0.066 (<i>P</i> = .610)	0.035 (<i>P</i> = .683)
HbA1c (%) (not adjusted correlations)	0.055 (<i>P</i> = .755)	-0.137 (<i>P</i> = .280)	0.018 (<i>P</i> = .831)

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment; UAG, unacylated ghrelin.

Correlation coefficients between UAG and anthropometric and hormonal parameters and partial correlations (‡) between UAG and metabolic parameters in obesity stratified subgroups: Pearson correlation when both variables have normal distribution (*) and Spearman correlation otherwise. Significant *P* values are in bold.

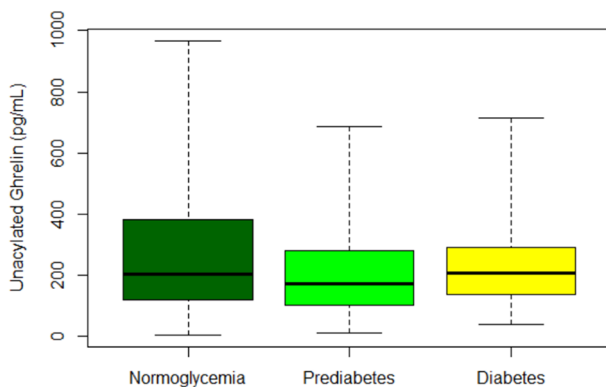


Figure 2. Unacylated ghrelin boxplots according to glycemic status in obese women: normoglycemic (*n* = 63), prediabetic (*n* = 63), diabetic (*n* = 19).

might reflect UAG decrease.^{20,26-29} Furthermore, some authors have suggested that UAG levels are influenced by the degree of adiposity.^{20,30} In the present study, we have confirmed that obese women show significantly lower levels of UAG and a negative association was found with BMI (but only among obese women). That is in accordance with the hypothesis that excessive fat mass can induce a decrease in UAG levels. However, no significant difference was shown among obesity

class subgroups. That could be the result of a fat mass threshold level that, when attained, do not results in further reduction of UAG levels. However, other mechanisms could contribute to these results.

Several studies suggest that diabetes and obesity are linked to a decrease in UAG.²⁶⁻²⁹ As obesity is commonly associated with prediabetes and diabetes, it was important to clarify whether the lowering of UAG levels was associated with dysglycemia from an adiposity-independent fashion. In our study, no correlation was found between UAG and glucose, insulin, insulin resistance, and metabolic control parameters showing that UAG plasma levels are not associated to glucose metabolism status. In spite of some studies suggesting of a metabolic benefit of UAG, results are still inconsistent and contradictory.^{16,19-21,31,32}

In our study, no difference in UAG levels was found among glycemic status subgroups (normoglycemia, prediabetes, and diabetes), and no association was found with glucose, insulin, HOMA-IR, or HbA1c values. Facing these results, we may conclude that UAG does not associate with glucose homeostasis in obese women.

More intervention studies will be necessary to better understand the action (if any) of UAG in obesity and on its comorbidities, namely in what concerns glucose metabolism.

Table 4. Correlation coefficients between UAG and anthropometric, hormonal, and metabolic parameters according to glycemic status.

VARIABLE	NORMOGLYCEMIA N = 165	PREDIABETES N = 63	DIABETES N = 19
BMI (kg/m ²)	-0.230 (P < .001)	-0.220 (P = .078)	-0.211 (P = .402)*
Total body weight (kg)	-0.213 (P = .001)	-0.287 (P = .020)	-0.246 (P = .325)*
Total body fat (kg)	-0.208 (P = .001)	-0.318 (P = .010)	-0.279 (P = .263)*
Percentage of body fat (%)	-0.189 (P = .002)	-0.286 (P = .021)	-0.142 (P = .575)*
Waist circumference (cm)	-0.221 (P < .001)	-0.211 (P = .910)	-0.280 (P = .260)*
Hip circumference (cm)	-0.199 (P = .001)	-0.189 (P = .131)	-0.289 (P = .244)*
Waist-to-hip ratio	-0.189 (P = .003)	-0.207 (P = .098)	-0.188 (P = .454)*
Fasting Insulin (mg/dL)‡	0.002 (P = .971)	-0.107 (P = .408)	0.049 (P = .857)*
Fasting Insulin (mg/dL) (not adjusted correlations)	-0.122 (P = .053)	-0.107 (P = .408)	0.048 (P = .851)*
Fasting glucose (mg/dL)‡	-0.051 (P = .426)	-0.030 (P = .814)	-0.099 (P = .715)
Fasting glucose (mg/dL) (not adjusted correlations)	-0.031 (P = .619)	-0.016 (P = .902)	0.286 (P = .249)
HOMA-IR‡	-0.007 (P = .915)	-0.007 (P = .915)	0.023 (P = .934)*
HOMA-IR (not adjusted correlations)	-0.128 (P = .042)	-0.138 (P = .285)	0.022 (P = .931)*
HbA1c (%)‡	0.092 (P = .149)	0.107 (P = .407)	0.026 (P = .925)
HbA1c (%) (not adjusted correlations)	0.049 (P = .445)	-0.015 (P = .904)	0.180 (P = .474)

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment; UAG, unacylated ghrelin.

Correlation coefficients between UAG and anthropometric and hormonal parameters and partial correlations (‡) between UAG and metabolic parameters in the 3 glycemic status subgroups: Pearson correlation when both variables have normal distribution (*) and Spearman correlation otherwise. Significant P values are in bold.

Authors' Note

The results were partially presented in the 19th European Congress of Endocrinology.

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Author Contributions

LV, MB and JSN participated in the design of the project; LV and MB participate in the evaluation of biochemical parameters; SN was involved in the selection and collection of the sample and evaluation of anthropometric parameters; CS contributed with the statistical analyzes, reviewing and editing. All the authors contribute to the manuscript and approved the final version.

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